

# Analysis of High Throughput Flow Cytometry Data using *plateCore*

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# Abstract

## Background

High throughput flow cytometry (FCM) studies are often run in a 96 or 384-well plate format, with different samples, controls, and antibodies-dye conjugates present on each plate. Analyzing a plate requires tracking the contents of each well, matching sample wells with control wells, gating each well/channel separately, making the appropriate plots, assessing quality, and finally aggregating the results from multiple plates to make experiment level conclusions. This process can be a significant task using traditional point-and-click software packages, even when multiple instances are deployed. We developed *plateCore* as an R/Bioconductor packaged to make processing and analysis of large, complex datasets easier.

## Methods

*plateCore* was used to analyze the data from a BD FACS CAP screening experiment where 5 Peripheral Blood Mononucleocyte Cell (PBMC) samples were assayed for 189 different human cell surface markers. This same dataset was also manually analyzed by a cytometry expert using the FlowJo data analysis software package (TreeStar, Ashland OR).

## Results

Positive markers identified using *plateCore* are in good agreement with those found using manual analysis.

## Conclusions

*plateCore* provides a reproducible, objective platform for analyzing high throughput FCM experiments. The R/Bioconductor implementation allows bioinformaticians and statisticians access to the data, which should further the development of automated analysis methods.

# Introduction

While there are a number of different software packages available for analysis of FCM data, these programs are often ill-suited to the development of new methods needed for analyzing high-throughput FCM studies. Flow Cytometry High Content Screening (FC-HCS) experiments generate large volumes of data, and a systematic approach to preprocessing, gating (i.e. filtering), and summarizing results is needed for robust analyses. Automation of these steps would allow analysis pipelines to be robust, objective, and match the high-throughput capacity of modern cytometers. Unfortunately, current approaches to FC-HCS analysis are semi-automated at best, and they often require significant subjective and error-prone manual intervention to identify cells of interest (Maecker et al., 2005). It is therefore desirable to develop programmatic approaches to process FCM data.

FCM packages available through the Bioconductor (Gentleman et al., 2004) project provide an open platform that can be used by cytometrists, bioinformaticians, and statisticians to collaboratively develop new methods for automated FC-HCS analysis. The basic data processing tools for importing, transforming, gating, and organizing raw FCM data are in the *flowCore* package (Hahne et al., 2009), and the visualization functions are in *flowViz* (Sarkar et al., 2008). The Bioconductor model for FCM data analysis facilitates the development of new analysis methods, since the overhead associated with accessing and visualizing FCM data is handled by *flowCore* and *flowViz*. The availability of *flowCore* and *flowViz* has enabled the creation of new tools for quality assessment of large FCM experiments, such as *flowQ* (Gentleman et al.), and model-based clustering and automated gating, such as *flowClust* (Lo et al., 2008).

We have developed an R package (*plateCore*) that also takes advantage of the functionality in *flowCore* and *flowViz* to create methods and data structures for processing large, plate-based FCM datasets. *plateCore* is not designed to be a GUI driven end-user tool, but rather to help develop a standardized platform for the analysis of FC-HCS data. These analyses often represent a collaborative effort between cytometry experts who generate the data and the quantitative individuals who help deal with the large volume information. In order for this collaboration to work, the cytometrists must have confidence in the results of the automated analysis. To this point, we demonstrate the equality of our results to those produced by an expert cytometrist using FlowJo.

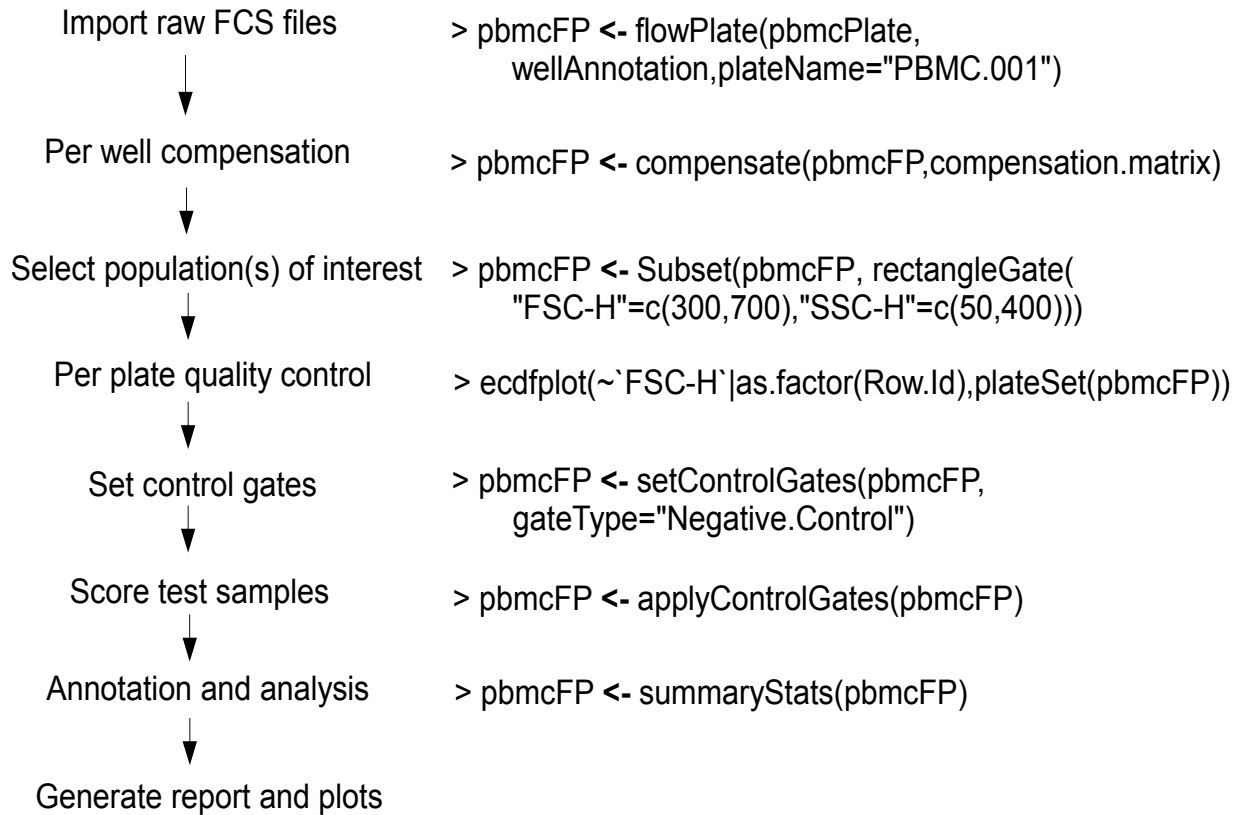


Figure 1: Typical FC-HCS plate workflow on the left and corresponding steps from a PBMC lymphocyte *plateCore* analysis on the right. Compared to analyses performed using existing GUI FCM tools, *plateCore* can reduce the level of subjectivity associated with creating the negative control gates and also makes it easier to aggregate multiple plates into an experiment level object for visualization and reporting. Providing *plateCore* scripts along with the raw FCM data for FC-HCS experiments helps to ensure that analysis is transparent and reproducible. ( Need to label each of the columns (manual analysis; plateCore workflow). is the last step in plateCore column lined up OK? Is it supposed to match last 2 steps in left column, just the last step? - Ryan)

# Materials and Methods

## Data

BD FACS CAP was designed as a cell characterization tool to screen for the presence a large number of different human cell surface markers. In this study, previously frozen PBMC samples from two donors were analyzed on a BD FACS Calibur using BD FACS CAP staining. The analysis was performed on 96-well plates with 189 different antibodies arrayed three per well in 63 test wells, along with 30 isotype control wells and three unstained controls. The complete list of BD FACS CAP antibodies can be found at [http://www.bd.com/technologies/discovery\\_platform/BD\\_FACS\\_CAP.asp](http://www.bd.com/technologies/discovery_platform/BD_FACS_CAP.asp). FCM files for the 5 plates (two for Donor 1 and three for Donor 2), are available for download from <http://www.ficcs.org>.

## Analysis

The goal of the PBMC FACS CAP study was to look for positive staining for the 189 different cell surface markers in lymphocytes. The *plateCore* scripts used to perform the analysis are provided in supplementary materials. Briefly, the FCM files were first processed using a combination of static gates(`rectangleGate`) and data driven gates (using `norm2filter` and *flowCore*) to pick out the lymphocytes in the forward (FSC) and side scatter (SSC) (%correct? - Ryan) channels. The quality of the data was then assessed by looking for fluidic events such as bubbles, pressure drops, or large aggregates that can shift the baseline fluorescence readings. Fluidic events can often be identified by plotting the empirical cumulative density (ecdf) plots of FSC values for each well, and looking for distributions shifted relative to other wells (Le Meur et al., 2007). Based on the ecdf plots, several wells were further investigated by cytometry experts who determined that the shifts were in an acceptable range. Next the threshold between positive and negative cells were determined using the isotype controls, which provided a gross estimate of non-specific binding in the primary antibodies. One-dimensional gates were created using the isotype thresholds, and these gates were applied to identify cells that are positively stained for each marker.

An example of the progression from raw FCM data files to a completed *plateCore* analysis is shown in Figure 1. List mode FCS files for a single plate were read into a `flowSet` using *flowCore*, and then a `flowPlate` was created by integrating the plate annotation file with the `flowSet`. The `flowPlate` was then compensated, data quality was assessed, and gates were set according to a negative control. These control gates were then applied to test wells to find cells that had specific staining in channels of interest.

In addition to *plateCore*, the five PBMC plates were also analyzed using FlowJo. First, an analysis template is created where test wells and their corresponding isotype control well were assigned to one of 30 groups. Wells in each group had similar sets of antibody-dye conjugates, and the expression threshold (*i.e.*, isotype gate) was initially

set using the isotype control well. Data for each plate was imported into FlowJo using the template and lymphocytes were selected using a morphology (FSC-SSC) gate. Event data for the isotype well was then visualized on a log scale, and the expression threshold for each stained channel was set by picking a value that lies above the bulk of the events. For BD FACS CAP, the isotype gate is typically (correct? does it always have to be 1% exactly? If so reference a published protocol or say according to manufactureres intructions or something - Ryan) set so that less than 1% of the events in the isotype well are above the threshold. These gates were then applied to the test wells. The threshold was moved up or down based on positive test wells. (%explain this better, when moved, under what conditions, and why - Ryan) The percentage of cells above the threshold for each of the 189 antibodies was then exported to a separate spreadsheet for each plate. (%and then what? Some kind of comparison was made .... From Ryan)

## Results

### *plateCore*

The five PBMC plates were analyzed using the approach shown in Figure 1. Results were stored in `flowPlates`, which are data structures that contain a description of the plate layout, morphology gated (FSC-SSC) events, and parameters values for the the negative control gates (*i.e.*, isotype gates). Event level data can be visualized using plotting functions from *plateCore* and *flowViz*. Additionally, results from different plates can be aggregated, making it easier to compare results from different plates and to create the complex reports required to summarize results from 189 different markers.

A marker was classified as positive for BD FACS CAP was defined as having more than 10% of events above the isotype gate. Since antibody concentrations used in BD FACS CAP are designed to screen a number of different cell types, the concentrations are not necessarily optimal for these PBMC samples. The 10% cutoff is an empirically determined threshold (data not shown) used to select markers for further analysis, including single color titration and competition experiments to confirm that the marker is present and staining is specific. Markers that are highly positive ( $\geq 90\%$ ) are usually confirmed in follow-up studies, while markers that are low positives ( $\leq 15\%$ ) are often the result of non-specific staining. Also, these percentages refer to the fraction of events above the isotype threshold, but this does not necessarily imply heterogeneous staining in multiple populations. In this study 109/189 markers were positive on at least one plate.

Since the majority of antibodies on the BD FACS CAP staining plate are known to bind different leukocytes, it is not surprising that a large fraction would be identified as positive on PBMCs. Markers such as CD44, CD45, CD47, and CD59 are broadly expressed on lymphocytes and were highly positive ( $>99\%$ ) in this study.

The difficulty in determining whether a dim marker is expressed can be seen in Figure 2, where samples with Median Fluorescence Intensity (MFI) (%reference to what

MFI is? - Ryan) ratios between 1 and 10 ranged from 0 to 100% percent positive. If the isotype gate is near the MFI of the test well signal, then estimates of the percentage of positive cells will be unstable. (%explain why - Ryan)

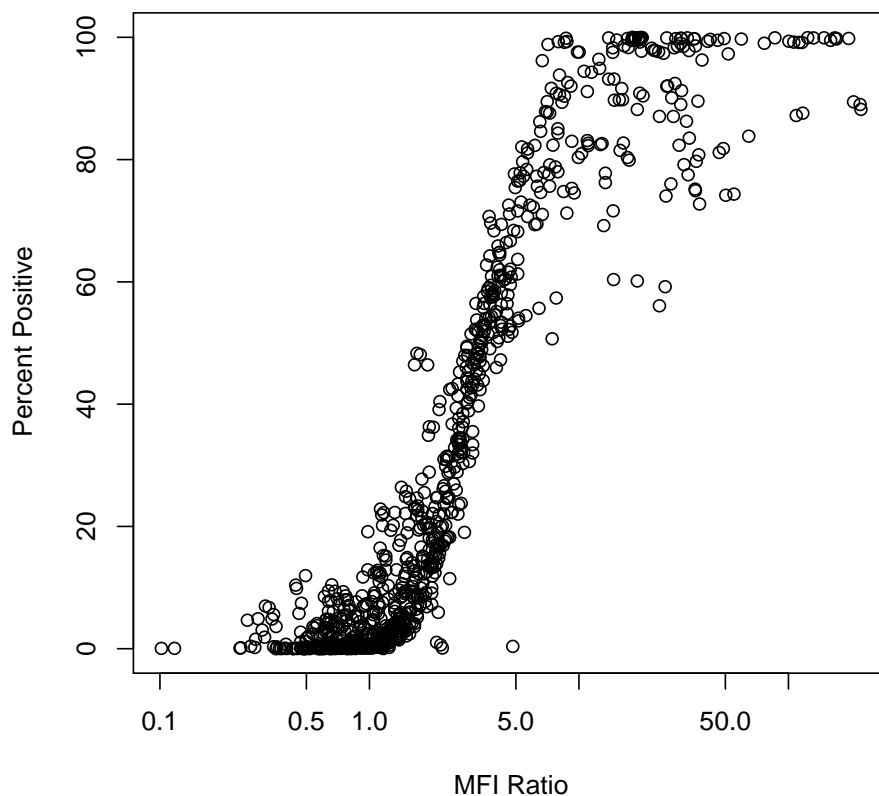


Figure 2: Plot of the Median Fluorescence Intensity (MFI) ratio for each of the 189 antibody-dye conjugates versus the percentage of positive events identified using plate-Core from the five PBMC plates. The MFI ratio refers to the ratio of the test well MFI to the corresponding isotype control on a linear scale. Cell populations with MFIs that are close to the isotype control are often split by the isotype gate, reflecting the range of percent positive values for MFI ratios between 1 and 10. (%label axis better - % positive whats - Ryan)



## Comparison to FlowJo Results

Automating the creation and modification of isotype gates made by cytometrists analyzing BD FACS CAP data using FlowJo is challenging. Cytometrists adjust gates based on expert knowledge about the performance of specific antibody types and dyes, or after identifying positive test samples. The automated approach employed in *plateCore* determines the threshold using isotype controls. The gate ( $G_{ij}$ ) for isotype  $i$ , channel  $j$  is set according to:

$$G_{ij} = \max(99\text{th}_{ij}, \text{MFI}_{ij} + 4\text{MAD}_{ij}), \quad (1)$$

where  $99\text{th}_{ij}$  is the 99th percentile for the fluorescence signal and MAD is Median Absolute Deviation on a linear scale. While this simple, non-parametric method works surprisingly well for BD FACS CAP, advances in model-based clustering methods, such as those in *flowClust*, should lead to future performance improvements in automated gating. Comparisons of the *plateCore* and FlowJo analysis are shown in Figure 3.

Examining in detail at one case where the *plateCore* and FlowJo methods disagree, such as CD98 on plate 9208 (Figure 4), highlights situations where our automated gating approach will fail. In this case the marker is positive (86% by FlowJo) (%86% what? This is confusing as you said something over 10% is labelled positive (I think) so 86% positive is confusing - Ryan) but is called negative (0%) (%0% what (same problem) - Ryan) by *plateCore*. The isotype control (well H08 in Figure 4) has >1% of its events in the FL1-H channel (%I know caliburs can't be changed, but can you give this a meaningful label for those that aren't used to the platform - Ryan) that are above the main population, resulting in the automated gate being set at the 99th percentile instead of the  $\text{MFI} + 4\text{MADS}$ . Fortunately, this type of mistake can also be detected by looking the MFI ratio plot in Figure 2. CD98 for plate 9208 has an MFI ratio of 5 and a percent positive value of 0%, while other markers with a similar MFI ratio have values near 70%. Percent positive values that lie far from the sigmoid curve in Figure 2 should be evaluated manually.

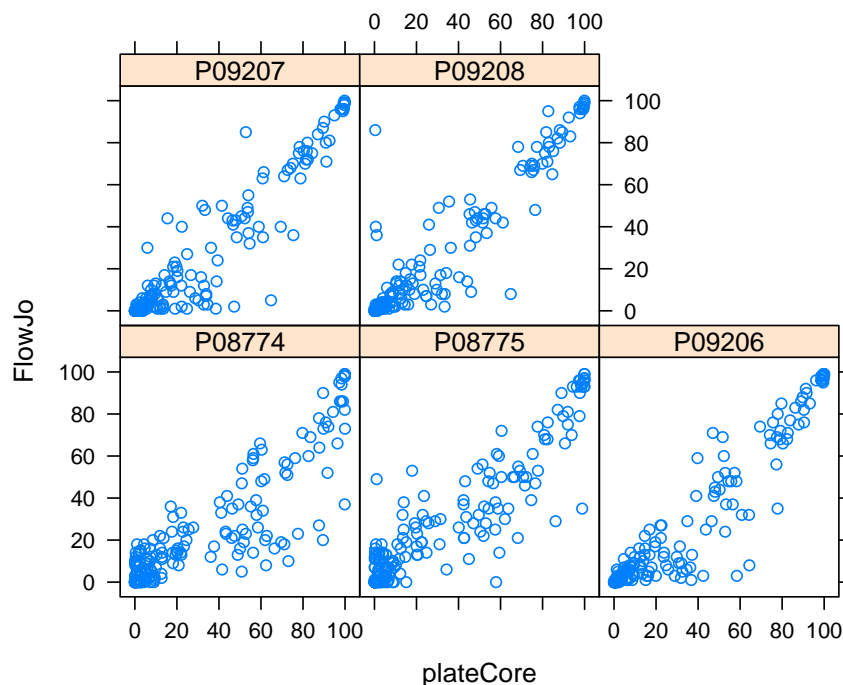


Figure 3: Percent positive results for 189 BD FACS CAP markers analyzed using either *plateCore* or FlowJo for the 5 PBMC plates. Markers that varied the most between the the two methods tended to have intermediate percent positive values (30% to 70%), reflecting the difficulty of gating populations whose MFI is near the isotype threshold. (%need to say what the P0907 labels mean, need mroe informative labels than just "plate Cote and FlowJo (maybe jsut stick "analysis) at the end. Again its confusing what positive vs 30% positive is. You said there was a threshold at 10% to get the label of positive. – Ryan)

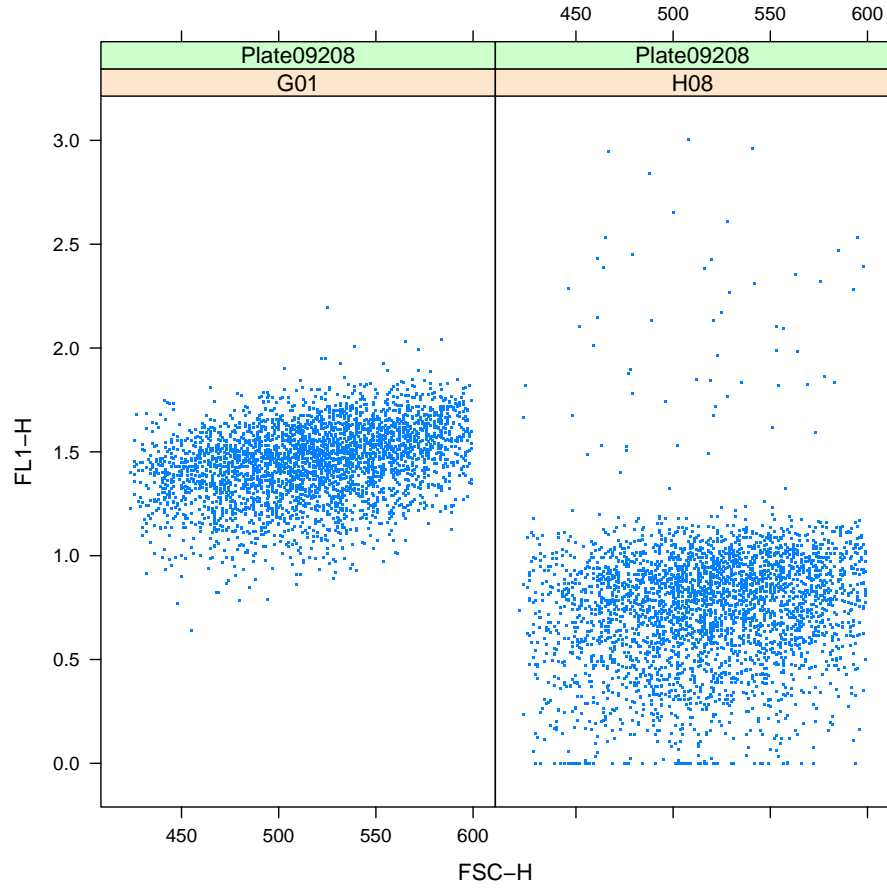


Figure 4: Dotplot for CD98 in well G01 and its isotype control well H08 for plate 9208. FlowJo (86%) and plateCore (0%) reported dramatically different percent positive values for this marker. The difference was caused by the isotype well having >1% of the events above the main population, which resulted in the automated gate being set at the 99th% instead of the MFI+4MADs. (indicate that G01 is a well ID PLate0909 is a plate ID - Ryan)

## Donor Variation

A common goal for BD FACS CAP screens is to identify markers that show variation in expression levels among cells isolated from different donors. Variation in estimates of the percentage of positive cells can be modeled using a binomial, where markers that are clearly positive or negative (0 or 100%) have little variation and those near the isotype gate (50%) are highly variable. Unfortunately, the power to detect differences in this study is limited since there are only 2 donors and the level of replication is low (2-3 plates per donor). Figure 5 shows the expected and observed variation in the percentage of positively stained cells for the 189 markers. Discounting CD98, which had an unusual isotype control, the markers with the most variation (S.D. near 30%) were CD85, CD97, CD154, CD184, and CD252. Since CD85, CD97, CD154, and CD252 are associated with activated T-cells, it is tempting to speculate that this difference represents biological variation between the two donors as opposed to technical differences in the processing and gating of the cells.

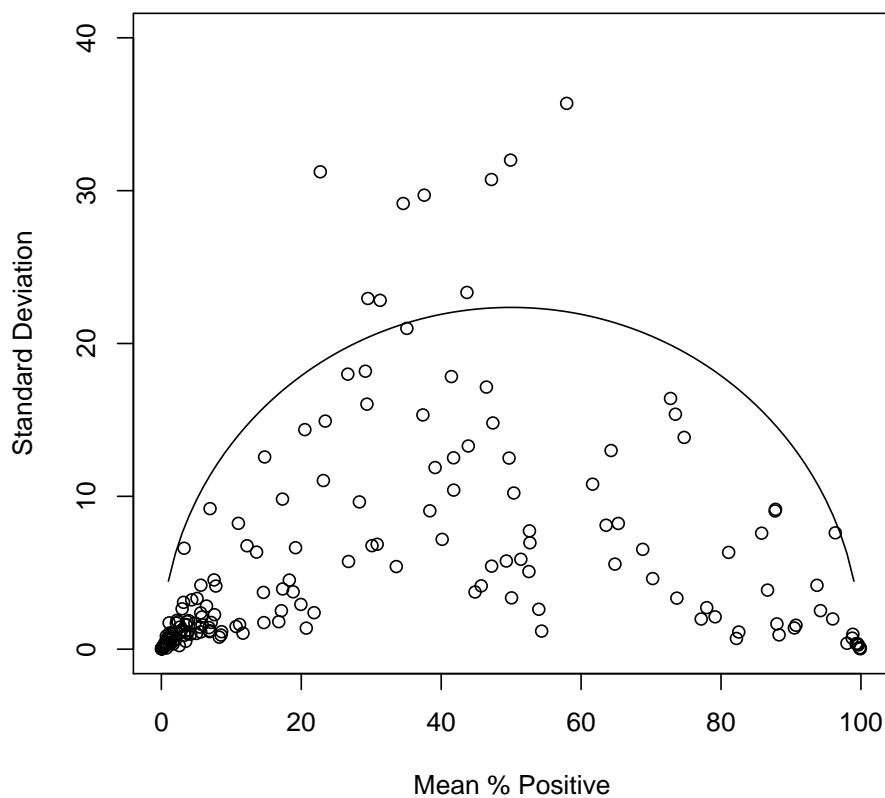


Figure 5: Scatterplot showing the mean percentage of positive cells for each positive marker versus the observed (circles) and expected (line) sample standard deviation. Expected values follow a binomial distribution with  $n=5$ . Markers that lie above the expected line will (%were? - Ryan) be further evaluated using titration and competition experiments to see if these results represent real variation between the two donors. The curve in the expected line reflects the difficulty in gating samples whose median signal (MFI) is near the isotype cutoff, since the percentage of positive cells calculated can shift dramatically with small changes in the gate. (standard deveiation of what? mean % positive of what? - Ryan))

## Discussion

Our approach to this PBMC BD FACS CAP study relied on processing the raw data in parallel using both FlowJo and *plateCore*. FlowJo allowed the cytometrists to thoroughly investigate individual wells, and gave them confidence that the *plateCore* results were correct (see Figure 3). Using *plateCore*, we were able to reduce the level subjectivity in setting isotype gates, eliminate mistakes associated with manual export and merging of plate output, and automate the creation of plots and data quality reports that summarized the experiment. Additionally, the *plateCore* scripts and experimental annotation can be shared with other cytometry groups, allowing them to reproduce our analysis.

In addition to subjective gating, the lack of a standard format for describing large FCM experiments also makes it difficult for anyone other than the original experimenter to replicate an analysis. (You could add something about MIFlowCyt here as well, as even if people had a format to follow, unless they put all the data in necessary to understand, just having a format isn't enough - Ryan) The development of mechanism to bundle experimental metadata descriptions with FCS data files should make it easier to access metadata in future FCM studies, but currently this information is typically provided either as spreadsheet or a pictorial layout of a 96 well plate. Since the creation of **flowPlate** requires users to make a standard sample annotation file, plate layouts from *plateCore* can then be easily shared along with the raw FCS2.0/3.0 files. The standard format for *plateCore* sample annotations provides a convenient way to manage the plate metadata associated with complex FC-HCS experiments.

While this same analysis can be performed relatively quickly in other FCM software packages, it can be difficult to reproduce the gating decisions made by a single expert user. (Expand - Ryan)

Markers that are expressed on a small subset of lymphocytes, or markers that are dimly expressed, would not be found with this screening approach. (Expand - Ryan)

Since, BD FACS CAP was designed as screening tool to identify markers for further analysis, false negatives were a bigger concern than false positives. The *plateCore* settings were chosen to err on the side of calling samples positive. (Why, make it clear to uninformed readers by stating explicitly? what about for other studies? more discussion here - Ryan)

The complexity of large FCM experiments, like BD FACS CAP, highlight the difficulty of applying existing FCM analysis platforms to high-throughput studies. Generating and interpreting results from this PBMC study required extensive collaboration between flow cytometrists, bioinformaticians, and statisticians. At various points in the analysis, each group needed to access the raw data, annotation, and details about the experimental design. Providing this access using stand-alone FCM platforms is expensive in terms of the price of multiple software licenses and in time spent training statisticians and bioinformaticians to use the programs. Fortunately the Bioconductor FCM packages are modeled on standard data structures used for microarrays, which should al-

ready be familiar to most quantitative individuals working on high-throughput biological problems. We found that *flowCore*, *flowViz*, and *plateCore* provided an open analysis platform that facilitated communication between the flow cytometrists generating the data, and the computational experts analyzing the data.

## References

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